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de Wilde, MC; Farkas, E; Gerrits, M; Kiliaan, AJ; Luiten, PGM; Wilde, Martijn C. de; Kiliaan, Amanda J.

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## Research report

# The effect of n-3 polyunsaturated fatty acid-rich diets on cognitive and cerebrovascular parameters in chronic cerebral hypoperfusion

Martijn C. de Wilde<sup>a</sup>, Eszter Farkas<sup>a,c</sup>, Marjolein Gerrits<sup>a</sup>, Amanda J. Kiliaan<sup>b</sup>,  
Paul G.M. Luiten<sup>a,\*</sup>

<sup>a</sup>Department of Animal Physiology, Workgroup of Molecular Neurobiology, University of Groningen, P.O. Box 14, 9750 AA Haren, The Netherlands

<sup>b</sup>Numico Research, Wageningen, The Netherlands

<sup>c</sup>Department of Anatomy, Albert Szent-Györgyi Health Science Center, Faculty of Medicine, University of Szeged, Szeged, Hungary

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## Abstract

Western diets consist to a large part of n-6 polyunsaturated fatty acids (PUFAs). These n-6 PUFAs and their conversion products favor immune and inflammatory reactions and compromise vasoregulation, which can contribute to the development of dementia. Recent epidemiological studies associated dementia, particularly the type accompanied by a vascular component, with high, saturated dietary fat intake. Conversely, high fish consumption (a source of long chain n-3 PUFAs) was related to a reduced risk for cognitive decline. Therefore we studied the effects of long chain n-3 PUFAs in rats with bilateral occlusion of the common carotid arteries (2VO), which mimics cerebral hypoperfusion, a risk factor for dementia. Male Wistar rats received experimental diets with a decreased (n-6)/(n-3) ratio from weaning on. At the age of 3 months, the animals underwent 2VO surgery. The rats were tested in the elevated plus maze, an active avoidance paradigm and the Morris water maze (at different survival times). Following behavioral testing, the animals were sacrificed at the age of 7 months. The frontoparietal cortex was analyzed for capillary ultrastructure with electron microscopy. No effects of cerebral hypoperfusion or diet were found on elevated plus maze and active avoidance, while spatial memory in the Morris maze was compromised due to cerebral hypoperfusion under placebo dietary conditions. n-3 PUFA supplementation in combination with extra additives improved the performance of the 2VO animals. The number of endothelial mitochondria, as well as the ratio of microvessels with degenerative pericytes appeared to be lower due to long chain n-3 PUFAs. These results may indicate an improved condition of the blood–brain barrier.

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**Topic:** Nutritional and prenatal factors

**Keywords:** Blood–brain barrier; Dietary; Cerebral hypoperfusion; Electron microscopy; Polyunsaturated fatty acid; Spatial learning

## 1. Introduction

Long chain polyunsaturated fatty acids (PUFAs) are essential for the optimal development of the organism and the functional development of the brain in particular, since PUFAs form significant structural components of the cerebral phospholipid membranes. Both n-3 and n-6 PUFA families originate from vegetable oil whereas fish oil is known as a rich source of the long chain n-3 type PUFAs.

A prominent member of the n-6 PUFA family, linoleic acid LA (LA; 18:2n-6) serves as the precursor for arachidonic acid (AA; 20:4n-6), a strongly bioactive molecule. On the other hand, the n-3 type  $\alpha$ -linolenic acid ( $\alpha$ LA; 18:3n-3) is elongated to eicosapentaenoic acid (EPA; 20:5n-3), and is processed further into docosahexaenoic acid (DHA; 22:6n-3), which can effectively influence neuronal membrane fluidity.

The brain is an organ generally well-protected against external blood-borne influences, but the cerebral fatty acid composition can be extensively modulated by dietary lipids [1]. Apparently, the concentration of DHA in neuronal membranes is especially important since this PUFA is

\*Corresponding author. Tel.: +31-50-363-2359; fax: +31-50-363-2331.

E-mail address: [p.g.m.luiten@biol.rug.nl](mailto:p.g.m.luiten@biol.rug.nl) (P.G.M. Luiten).

taken up by the brain in preference to other fatty acids [14]. Furthermore, changing the dietary (n-6)/(n-3) ratio effectively modifies the fluidity of phospholipid membranes [16]. The influences of dietary PUFA composition are, however, not restricted to structural changes in the plasma membranes but can also be reflected in behavioral and cognitive functioning [17,29]. In particular, long chain n-3 PUFA supplementation to laboratory animals has been demonstrated to improve spatial learning capacity. Providing rats with n-3 PUFAs resulted in a lower percentage of rats unable to locate the escape platform in the Morris water maze, and shorter escape latencies indicative of improved working memory, when compared to control animals [15]. Further, Gamoh et al. [12] reported that chronic administration of DHA to animals brought up on a long chain n-3 PUFA deficient diet resulted in improved reference memory-related learning. These rats made less errors compared to animals treated with vehicle in the eight-arm radial maze. Conversely, rats nourished with diets deficient in DHA showed longer escape latencies and delayed acquisition in the Morris maze task compared to animals supplied with a diet containing a sufficient amount of DHA [21]. These studies suggest that the n-3 content of the diet and therefore the level of DHA in brain phospholipids is very important for learning ability. In summary, animals with higher dietary and brain levels of DHA were shown to have a better performance in spatial learning tasks compared to animals with abnormally low n-3 PUFA levels.

Spatial memory can be compromised by several pathophysiological factors, such as a chronically reduced cerebral blood flow [11]. Studies imposing permanent bilateral occlusion of the common carotid arteries (two-vessel occlusion, 2VO) to model cerebral hypoperfusion as it occurs in aging and dementia, have reported declining spatial learning capability [11]. When rats with 2VO were compared with SHAM operated controls in the Morris water maze, they showed increased latencies in locating the platform and spent a lower percentage of time in the platform area after platform removal [5]. This observation demonstrated both working memory and reference memory impairment.

Besides compromised spatial memory due to chronic cerebral hypoperfusion, reduced cerebral blood flow (CBF) was also shown to cause damage to the ultrastructure of hippocampal capillaries [5]. The degenerated capillaries displayed similar sorts of microvascular artifacts as found in aging rats and demented humans, namely an increased percentage of microvessels with basement membrane deposits and degenerative pericytes [9,11]. Moreover, the observed capillary basement membrane pathology positively correlated with impaired spatial memory, meaning a higher degree of basement membrane deposits coinciding with impaired spatial learning [5].

The present study was designed to test the potential beneficial effects of dietary long chain n-3 PUFAs in rats

with bilateral occlusion of the common carotid arteries (2VO), which creates cerebral hypoperfusion, a risk factor for dementia. Three diets, one placebo control and two experimental diets supplemented with a decreased (n-6)/(n-3) PUFA ratio were fed to rats from weaning till the age of 7 months. Our main focus was to visualize whether dietary long chain PUFAs could compensate the cognitive deficits and cerebrovascular breakdown induced by experimental cerebral hypoperfusion.

## 2. Materials and methods

### 2.1. Animals

Sixty, 30-day-old male Wistar rats were randomly assigned to three groups receiving different diets (20 animals per group). The animals were socially housed in cages of five, throughout the entire experiment. Food and water were available *ad libitum*. The weight of the animals was checked weekly.

### 2.2. Diets

The experimental rat diet was designed by Numico Research, Wageningen, The Netherlands. In the present experiment, the effects of two PUFA-enriched diets on behavior and cerebral microvascular integrity were compared to placebo. The placebo diet (Control diet) was essentially similar to the standard lab chow produced by Hopefarms (Woerden, The Netherlands). The three diets were identical with respect to the composition and amount of carbohydrates, proteins, minerals and caloric value, and were manufactured in the form of regular food pellets. The experimental diets (Diet 1 and Diet 2) were enriched by PUFAs, antioxidants, vitamins and particular extra additives. The lipid composition of the diets is listed in Table 1, the PUFA contents are shown in Table 2, and Table 3 indicates the types and amounts of the other additives. Diet 1 differed from the Control diet only in that Diet 1 contained additional PUFAs, where the (n-6)/(n-3) ratio was reduced to 1.17 compared to 4.35 in the Control diet. To stabilize the PUFA additions in Diet 1, antioxidants and vitamins were added (Table 3). Diet 2 was a complex diet enriched with PUFAs at an (n-6)/(n-3) ratio of 1.38,

Table 1  
The lipid composition of the experimental diets (g/100 g)

Component	Control	Diet 1	Diet 2
Soybean oil (Florin, Switzerland)	5	2.5	2.5
Marinol C45 (source of EPA and DHA) (Loders Crocklaan, The Netherlands)	–	2.15	2.15
Ropufa 50 (source of AA) (Roche, The Netherlands)	–	0.35	0.35

Table 2

The polyunsaturated fatty acid content of the experimental diets (g/100 g)

Fatty acid	Experimental diets		
	Control	Diet 1	Diet 2
18:2n-6 (LA)	0.640	1.321	1.661
20:4n-6 (AA)	0.000	0.118	0.118
Total n-6	0.675	1.476	1.820
18:3n-3 (ALA)	0.155	0.137	0.184
20:5n-3 (EPA)	0.000	0.589	0.589
22:6n-3 (DHA)	0.000	0.382	0.382
Total n-3	0.155	1.264	1.31
(n-6)/(n-3)	4.35	1.17	1.37

supplied with antioxidants and vitamins similar to Diet 1, and provided with other phospholipids and neurotransmitter precursors (see Tables 2 and 3 for details).

### 2.3. Surgery

At the age of 4 months, the common carotid arteries of half of the animals ( $n=10$ ) of each dietary group were bilaterally and permanently occluded to induce cerebral hypoperfusion (2VO). The other 10 animals per dietary group received the same surgical procedure but the actual occlusion of the arteries was not performed (SHAM animals). The surgical procedure was similar to the one reported by Farkas et al. [5]. Briefly, the animals were anesthetized by isoflurane gas. Via a longitudinal, cervical cut on the ventral surface of the neck, the left and the right carotid arteries were located by separating the muscle layers lateral to the trachea. The arteries were carefully separated from the vagal nerve, surgical threads were placed around them and tied up to create permanent occlusions. The wound was closed and the animals were observed during the first week of recovery. A week after surgery the overall survival rate was 88.3%.

Table 3

Additional supplements of the experimental diets (g/100 g)

Nutrient	Component	Control	Diet 1	Diet 2
Antioxidants and vitamins	$\beta$ carotene		0.02	0.02
	Flavonoids		0.2	0.2
	Folate	0.0004	0.001	0.001
	Selenium	0.000019	0.00004	0.00004
	Vitamin B6	0.00153	0.00172	0.00172
	Vitamin B12	0.00005	0.00012	0.00012
	Vitamin C		0.2	0.2
	Vitamin E	0.0063	0.3	0.3
Other	L-Acetylcarnitin			0.6
	Choline	0.15	0.15	0.4
	Phosphatidylcholine			0.2
	Phosphatidylserine			0.2
	Co-Q10			0.2
	Thiamin	0.002	0.002	0.2
	Tyrosine	0.944	0.944	1
	Tryptophan	0.232	0.232	1

### 2.4. Behavioral testing

All behavioral testing took place at the end of the animals' circadian activity phase. Three weeks after imposing 2VO all the animals were tested on the elevated plus maze to determine anxiety levels. The test was performed under dim light conditions. The animals were placed in the middle of the plus maze (50 cm above the floor), which had two closed and two open arms (45 cm long, 11 cm wide) the latter with a 1.5 cm high rim. The behavior of the rats was observed for five min. The time the animals spent on the open and closed arms was recorded. The time spent on the open arms was calculated as a ratio of the time spent on the open and the closed arms ( $o/o+c$ ), and taken as a measure for anxiety.

One week later associative learning capability was tested in the active avoidance test. The active avoidance box consisted of two compartments divided by a 1 cm high, visible plastic boundary. Both compartments were supplied with a grid floor, where a foot shock (0.3 mA, 3 s) could be alternately delivered. Each trial consisted of the delivery of a foot shock in the compartment the animal was staying, which was preceded by an discriminative stimulus, a ring of a buzzer for 3 s. The rats had to learn to avoid the footshock by jumping to the other compartment when the buzzer went on. First, the animals were placed in the box for 2 min to habituate to the new environment before starting the trials. Subsequently, each animal underwent 40 trials with a randomly chosen, variable inter-trial interval (20, 40 or 60 s). When the test was completed the number of active avoidances in clusters of five trials was counted and a learning curve was plotted.

Thirteen weeks after surgery, at the age of 7 months, the animals were tested in the Morris water maze. The Morris maze was used to test spatial memory. The maze consisted of a black, circular water tank (140 cm in diameter) and a square, hidden platform (20.5 cm $\times$ 20.5 cm,  $\pm$ 1 cm beneath water level). The water was 30 cm deep and had a temperature of  $26\pm 1$  °C. The water tank was located in an experimental room with various distal cues. The platform was always positioned 35 cm out of the rim of the pool on the same location with respect to the distal, visual cues. A video camera placed 2 m above the center of the pool was used to observe the animals' swimming pattern. The camera's signal was directed to a computer equipped with videotracking software (EthoVision 2.0, Noldus, Wageningen, The Netherlands). The software used divided the water maze into different zones. We defined a platform area, a circle directly surrounding the platform (48.5 cm in diameter). The rest of the pool was considered as the non-platform area. Swimming patterns were analyzed for the time necessary to locate the platform and the distance the animals had traveled before they reached the platform.

The animals were tested two times per day at the end of their activity period and start of their resting period, with a 3-h interval. There were four different starting points in the

water maze: two adjacent to the platform location and two opposite to it. For the first daily trial the animals were placed in the water at one of the close starting points and for the second trial per day they started at one of the far points. The animals were placed in the pool facing the rim and were given 2 min to locate the platform. When the rats could not find the platform within 2 min, they were gently directed to it. All animals were allowed to sit on the platform for 15 s before they were returned to their home cage. The acquisition phase of the test consisted of 6 days with 2 trials per day, where the average swimming distance of the 2 trials was calculated as an indicator of daily performance. Twenty-four hours after the last acquisition trial, the animals underwent a probe trial, where the platform was removed from the pool. The rats were allowed to swim for 1 min and the time spent in the platform area was registered as a measure for retention of the platform location.

### 2.5. Electron microscopic analysis of cerebrocortical capillaries

At the age of 7 months the animals were anesthetized with ether and given an overdose of pentobarbital (i.p. injection). The thorax was opened and the heart was prepared free of the pericardium. The rats were perfused with 100 ml saline containing 1 ml heparin followed by 400 ml fixative (2% paraformaldehyde, 0.05% glutaraldehyde, 0.2% picric acid in 0.1 M phosphate buffer, pH 7.4). The brains were carefully removed and placed in fresh fixative for electron microscopic analysis (1% paraformaldehyde and 2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4).

Coronal brain slices were cut at 50  $\mu\text{m}$  from the right hemispheres using a vibratome. The sections were collected in 0.1 phosphate buffer, routinely dehydrated and embedded in glycidic ether [5]. Samples of the frontoparietal cortex 0.2 mm rostral to Bregma (Paxinos and Watson) were selected and mounted on glycidic ether blocks. Then, ultrathin sections were cut with an ultramicrotome and five non-serial sections were collected on 200 mesh copper grids. The samples were contrasted with 5% uranyl-acetate and Reynolds lead-citrate solution, and examined with a Philips 201 transmission electron microscope. One grid per animal was examined, where an average tissue surface area of 0.125  $\text{mm}^2$  was analyzed. The surface area was determined with the help of the grid holes. The analysis included only grid holes completely covered by tissue, and only transversely cut capillaries were examined. All layers in the cortex were screened for microvascular basement membrane and pericyte pathology as defined in detail previously [10]. Briefly, capillaries showing local basement membrane thickening or collagen fibrils deposited in the basement membrane (fibrosis) were characterized as being abnormal. Degenerative pericytes were defined by membranous-appearing inclusion bodies or swelling. The

percentage of capillaries displaying any of the abnormal features was calculated from the total number of microvessels screened. Further, the number of transversely cut capillaries on a defined tissue surface was counted as a measure of capillary density and the lumen diameter of each vessel was determined. Finally, the number of endothelial mitochondria and tight junctions per capillary profile were also counted.

### 2.6. Statistics

Behavioral data were analyzed using repeated measurements analysis of variance (ANOVA). The results of the electron microscopic results were analyzed with a two-way ANOVA paradigm followed by the least significant difference post-hoc test.

## 3. Results

### 3.1. Behavior

No diet or cerebral hypoperfusion-related differences were found in the elevated plus maze. Animals of all groups spent the same percentage of time on the open arms and visited the open arms at the same frequency. Associative learning in the active avoidance paradigm was not affected by the 2VO condition or dietary supplements, either. All groups learned the test at an equal rate.

Fig. 1 shows the Morris water maze results. The SHAM animals from each dietary group learnt to locate the platform in the maze at a similar rate. All groups reached a plateau level of learning within the first 3 days of the acquisition phase ( $F=0.542$ ,  $P=0.589$ ). In the probe trial, no differences between the groups were found either. All the three groups spent significantly more time in the platform area compared to chance level as an indication of retention (Fig. 1A). Chance level is presented as a dotted line in the figure.

When comparing 2VO and SHAM animals in the Control dietary group (Fig. 1B), 2VO animals performed worse in the acquisition phase of the Morris maze than SHAM controls ( $F=5.493$ ,  $P=0.032$ ). The SHAM animals reached the plateau already on day 3 while 2VO animals needed almost the entire acquisition phase to reach the same level (day 5). In the probe trial, 2VO animals showed a significantly impaired retention of the platform location. Moreover, the 2VO animals did not differ from chance level in the probe trial, while SHAM animals did.

Analyzing the Diet 1 condition as presented in Fig. 1C, 2VO animals did not perform statistically inferior to SHAM in the learning phase of the Morris maze ( $F=3.938$ ,  $P=0.065$ ). However, inspecting the curves, it appeared that they still reached the plateau of learning with 1 day delay compared to SHAM. Similar to the 2VO animals on Control diet, the Diet 1 2VO animals did not

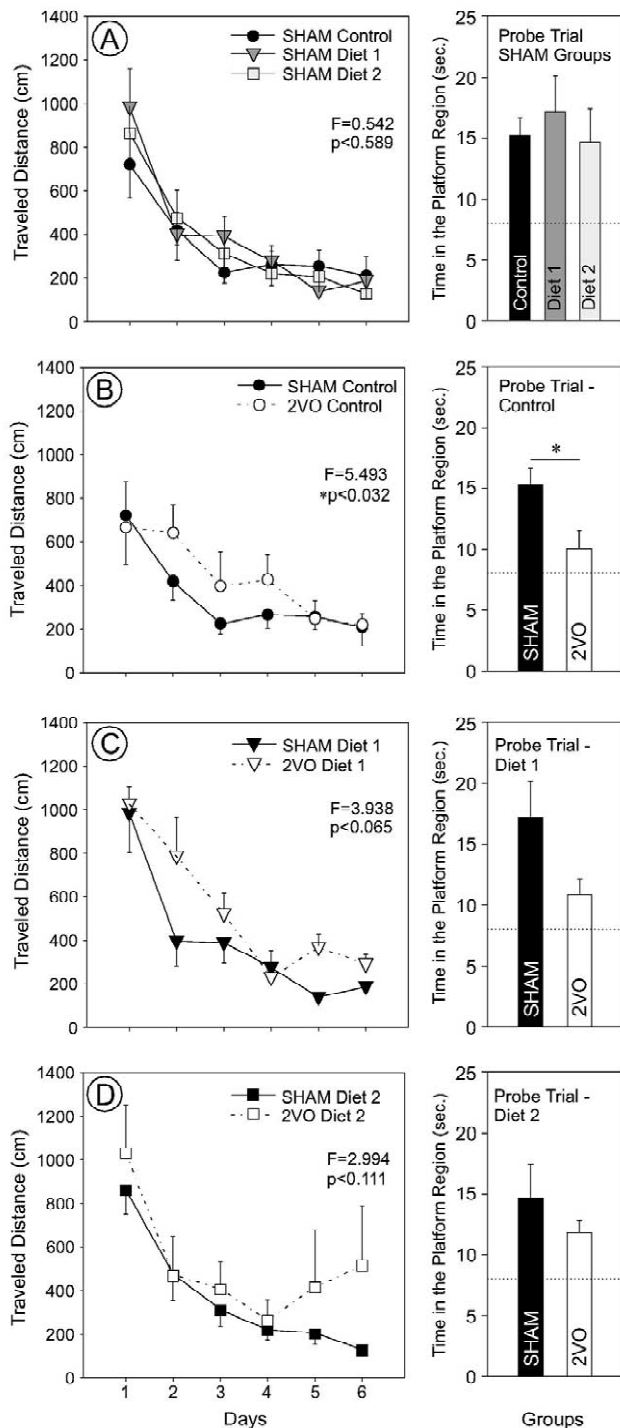


Fig. 1. Results of the Morris water maze spatial learning test. Abbreviations: 2VO: bilateral occlusion of the common carotid arteries, two-vessel occlusion; SHAM: sham-operated control. The error bars represent S.E.M. values.

differ from chance level in the probe trial, indicating that these animals were swimming in the pool randomly. Nevertheless, the significant difference between SHAM and 2VO under Control dietary condition could not be found in Diet 1 (Fig. 1C).

In case of Diet 2 (Fig. 1D), 2VO animals acquired the task at the same rate as did the SHAM animals ( $F=2.994$ ,  $P=0.111$ ), and they also reached the plateau of learning on the same day. Unlike on Control diet and Diet 1, the 2VO animals on Diet 2 performed significantly better than chance level in the probe trial.

### 3.2. Cerebral microvascular parameters

Neither cerebral hypoperfusion, nor dietary manipulations seemed to affect the percentage of microvessels with basement membrane aberrations, or the number of tight junctions per transverse capillary profile. No consistent differences between groups of 2VO and SHAM animals were detected in capillary density. Capillary density slightly increased due to Diet 2 supplementation, but the difference compared to the Control diet did not reach statistical significance (Fig. 2A). The capillary lumen diameters varied between group averages of 3.5–3.8  $\mu\text{m}$ , but were statistically the same for all groups (Fig. 2B).

2VO animals under placebo conditions tended to have a higher percentage of capillaries with degenerated pericytes than their SHAM controls, but the spread within groups was considerable (Fig. 3A). Two-way ANOVA revealed a

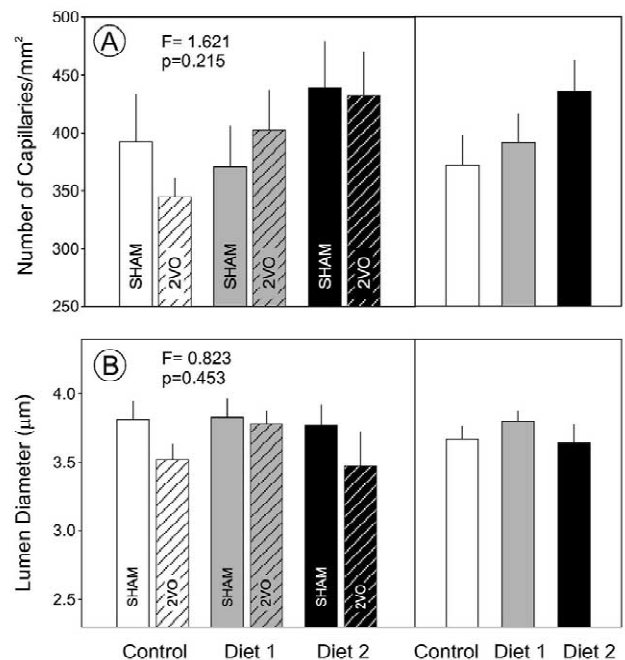


Fig. 2. Electron microscopic data of capillary density (A) and capillary lumen diameter (B) in the frontoparietal cortex. Abbreviations: 2VO: bilateral occlusion of the common carotid arteries, two-vessel occlusion; SHAM: sham-operated control. The  $F$  values in the left panels indicate the dietary effect as expressed by two-way ANOVA, the surgery effect was not significant. The right panel of the graph represents dietary effect of the two-way ANOVA analysis alone, where 2VO and SHAM animals of the same dietary group are combined. The error bars represent S.E.M. values.

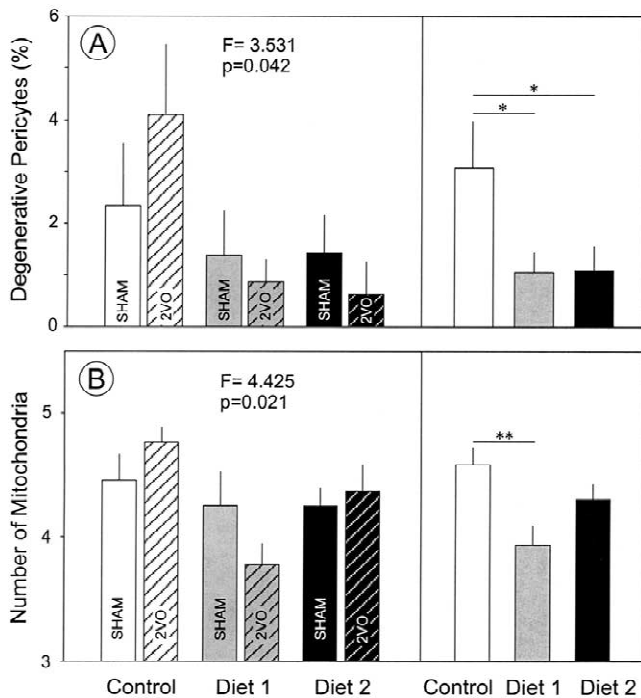


Fig. 3. Electron microscopic data of pericytic degeneration (A) and number of endothelial mitochondria (B) in the frontoparietal cortex. Abbreviations: 2VO: bilateral occlusion of the common carotid arteries, two-vessel occlusion; SHAM: sham-operated control. The  $F$  values in the left panels indicate the dietary effect as expressed by two-way ANOVA, the surgery effect was not significant. The right panel of the graph represents dietary effect of the two-way ANOVA analysis alone, where 2VO and SHAM animals of the same dietary group are combined. The asterisks stand for the dietary effect alone. The error bars represent S.E.M. values.

main dietary effect on the condition of pericytes, where both experimental diets decreased pericytic degeneration equally when compared to animals on the Control diet.

2VO animals of the Control dietary condition showed similar numbers of mitochondria present in the capillary endothelial cells compared to their SHAM controls (Fig. 3B). Two-way ANOVA analysis revealed a significant dietary effect on the number of endothelial mitochondria. Post-hoc analysis demonstrated that endothelial cells of Diet 1 fed animals contained less mitochondria compared to Control diet fed animals. The same tendency was obvious for Diet 2, but was not statistically significant. No difference existed between Diet 1 and Diet 2 animals.

#### 4. Discussion

This study aimed at investigating the potential beneficial effects of long chain n-3 PUFA supplementation on behavior and the anatomical integrity of cerebrocortical capillaries following experimentally induced chronic cerebral hypoperfusion.

##### 4.1. Behavior

To assess the PUFA effects on behavior, the animals were tested on the elevated plus maze for anxiety, in the active avoidance paradigm for associative learning abilities, and in the Morris water maze for spatial memory. Reduced cerebral blood flow or the experimental diets did not modify behavior on the elevated plus maze, neither did they affect escape behavior in the active avoidance task. The obtained results in the elevated plus maze confirmed the findings of Nakashima et al. [22] and Chalon et al. [4], who also described that levels of anxiety in the elevated plus maze were not affected by dietary supplementation with n-3 PUFAs.

The results here are in line with earlier findings that cerebral hypoperfusion had negative effects on spatial orientation in the Morris maze [5,23,24]. These latter studies revealed impaired Morris maze performance at various time points after surgery, including 3 months survival time also employed here [23,24]. Therefore, it can be stated that under control dietary conditions, the negative effects on spatial memory induced by experimental cerebral hypoperfusion could be reproduced.

Dietary supplementation did not affect spatial learning of healthy control animals (SHAM) in the water maze in our experiment. Studies with mice after dietary DHA treatment are in general agreement, that DHA improves spatial learning in the Morris maze [3,23,27]; however there was no effect of the (n-6)/(n-3) ratio [31]. Similarly, experiments tackling the spatial learning of rats in the water maze could not demonstrate after four generations that fish oil had more beneficial effects than a vegetable oil mixture [15]. Nevertheless, manipulations with the dietary (n-6)/(n-3) ratio revealed, that diets with an (n-6)/(n-3) ratio of 4 significantly improved Morris maze learning of rats [32]. Our experiment used diets with ratios of 1.17–1.38, which were below the level necessary to enhance Morris maze learning. In conclusion, the present study demonstrates that modestly increasing the dietary long chain n-3 PUFA ratio above the level in standard diet had no noticeable effect on the behavior of rats.

Our data further demonstrated that Diet 2—but not Diet 1—could restore Morris maze performance of 2VO rats to the corresponding SHAM level. Since Diet 2 contained several additions in comparison with Diet 1, it is most likely that the extra additives possibly in combination with PUFAs, rather than PUFA supplementation alone was responsible for the better Morris maze performance of the Diet 2 2VO rats.

##### 4.2. Cerebral microvascular parameters

Experimental cerebral hypoperfusion imposed for 13 weeks in the present experiment resulted only in a minor, not significant rise in capillary basement membrane pathology in 2VO animals compared to SHAM controls. Previ-

ous results with a longer post-operative period of 14 months showed remarkable damage to the cerebral capillaries in the form of basement membrane deposits [5]. The comparison of the two studies suggests that cerebral microvascular breakdown observed previously after 14 months of reduced blood supply to the brain does not arise shortly after the occlusion as an acute damage, but that it is gradually developing during a longer time span.

We have seen here that under control dietary conditions, capillary density in the frontal cortex was not altered after 13 weeks of experimental cerebral hypoperfusion whereas Sekhon et al. [26] reported an increased capillary density in the hippocampal CA1 area as a consequence of 20 weeks of cerebral hypoperfusion. Furthermore, angiogenesis and endothelial proliferation were described to occur in the adult brain under pathological conditions such as cerebral hypoxia/ischemia, particularly in the ischemic penumbra [19,25]. Since the mild, chronic cerebral hypoperfusion in our experiment did not induce microvascular proliferation, it is likely that acute cerebral ischemia (stroke) or a longer-term cerebral hypoperfusion must serve as pathophysiological initiators for increased capillary density.

The present cerebrovascular survey revealed that neither cerebral hypoperfusion nor dietary supplementation with PUFAs could affect capillary lumen diameters. While in vivo experiments involving the topical application of AA and DHA/EPA to the brain vasculature indicate that AA and DHA/EPA liberated in the vascular wall may modify vasoregulatory response [7,8,18], our data suggest that chronic supplementation with PUFAs does not alter the basal tone of the cerebral microvasculature.

Experimentally induced cerebral hypoperfusion was previously shown to result in increased pericytic degeneration in the rat brain [5]. In the present study, however, no statistical significance for augmented pericytic degeneration due to decreased blood flow was found. The inconsistency between the two experiments can find explanation in the length of chronic cerebral hypoperfusion, which was 12 months in the previous experiment [5] in contrast to 3 months in the present study. It is therefore possible that the longer duration of chronically reduced cerebral blood flow can proportionally increase pericytic degeneration.

Although pericytic degeneration was only slightly affected by chronic cerebral hypoperfusion here, dietary PUFA supplementation clearly decreased the ratio of degenerative pericytes. Pericytes were described to clean the extra-cellular space of debris via phagocytosis [30], a functional process that can also occur in oxidative stress. As a result, the pericytic cytoplasm can accumulate a large number of inclusion bodies considered as a perceptible feature of degeneration. Dietary n-3 PUFAs were demonstrated to reduce oxidative stress by competitively decreasing the metabolism of AA, which resulted in a lower production of free radicals [2,6]. Potentially lowering oxidative stress by n-3 PUFAs in our Diet 1 and Diet 2

could thus probably protect neuronal membranes, which may cause a reduced phagocytotic activity of pericytes. However, based on this reasoning, antioxidants such as vitamin E in the experimental diets could possibly also contribute to the protective effect on the pericytes.

The blood–brain barrier requires a substantial amount of energy for maintenance, which is provided to a considerable degree by the endothelial mitochondria. Investigating the interaction between mitochondrial energy production and PUFA treatment, mitochondrial ATP synthesis was found to be inhibited by increasing levels of AA in cerebral ischemia [28]. On the other hand, dietary n-3 fatty acid supplementation could eliminate mitochondrial dysfunction in cardiac tissue [20]. These experiments shed light on a positive influence of n-3 PUFAs on the metabolic efficiency of the mitochondrial enzymatic apparatus, but did not attempt to estimate changes in mitochondrial counts due to treatment. We observed here a decreased number of endothelial mitochondria after n-3 PUFA supplementation. In view of the above findings, we hypothesize that the n-3 PUFA content of Diet 1 and Diet 2 could possibly improve the actual capacity of individual mitochondria to provide ATP, therefore the energy demand of the blood–brain barrier could be satisfied by fewer endothelial mitochondria.

## 5. Conclusions

In summary, we suggest that increasing the dietary long chain n-3 PUFA ratio above standard values of intake has a less dramatic effect on memory than what the comparison of an n-3 PUFA deficient to an n-3 PUFA balanced diet would suggest [12,13,21]. Nevertheless, cerebral microvascular parameters such as the condition of pericytes and endothelial mitochondrial counts were altered by the increased n-3 PUFA ratio in the diet, which may indicate an improved condition of the blood–brain barrier.

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